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SEMI-CONSERVATIVE REPLICATION OF DOUBLE-STRANDED RNA BY A VIRION-ASSOCIATED RNA POLYMERASE

K.W. Buck

Department of Biochemistry
Imperial College of Science and Technology
London SW7 2AZ, U K

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SUMMARY

5-Bromo-UTP was found to replace UTP efficiently as a substrate for the virion-associated double-stranded RNA replicase of Penicillium stoloniferum virus PsV-S. The double-stranded RNA product of the replication reaction with 5-bromo-UTP as a substrate gave in equilibrium caesium sulphate density gradient centrifugation a single band with a buoyant density of 1.647 g/ml, consistent with that of a hybrid double-stranded RNA consisting of one brominated and one unbrominated strand. After the reaction none of the original unbrominated double-stranded RNA (buoyant density 1.606 g/ml) could be detected. It is concluded that replication of double-stranded RNA in virions of PsV-S takes place by a semi-conservative mechanism.

INTRODUCTION

Penicillium stoloniferum virus PsV-S is a small isometric double-stranded RNA (dsRNA) virus with a genome of two segments, dsRNA 1 (mol. wt. 1.11 x 10^6) and dsRNA 2 (mol. wt. 0.94 x 10^6), each of which is encapsidated separately in L2 and L1 particles respectively (1,2). Virus preparations contain also small amounts of M1 and M2 particles, which contain one molecule of virus single-stranded (messenger) RNA of mol. wt. 0.47 x 10^6 and 0.56 x 10^6 respectively, and substantial amounts of H particles, which contain either dsRNA 1 and dsRNA 2, together with single-stranded RNA (ss RNA) chains of various lengths. The capsids of all particles are constructed from the same polypeptides. H particles, but not M or L particles have RNA polymerase activity in vitro which catalyses the replication of dsRNA. The reaction terminates after one round of dsRNA replication and progeny dsRNA remains within virions giving rise to P1 and P2 particles which contain 2 molecules of dsRNA 2 and dsRNA 1 respectively (3). H particles

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are heterogeneous with a density range between those of L and P particles and contain intermediates of dsRNA replication, ranging from those in which the reaction has just been initiated (low density) to those in which it is almost complete (high density).

In the present communication it is shown, using density labelling, that dsRNA replication in virions of PsV-S proceeds by a semiconservative mechanism. This is the first report of semiconservative replication of dsRNA by a virion-associated RNA polymerase.

METHODS

Growth of the fungus, preparation and purification of virus and virus RNA, fractionation of virus preparations by velocity sucrose density gradient centrifugation and isopycnic caesium chloride density gradient centrifugation, were carried out as described previously (3).

RNA polymerase reactions. Reaction mixtures contained: 0.05 M-tris-HCl buffer, pH 8.0; 10 mM-$MgCl_2$; 1 mM-EDTA; 0.15 mM-GTP; 0.15 mM-CTP; 0.15 mM-^{3}H-ATP (specific activity 50 mCi/mmole), either 0.15 mM-UTP or 0.15 mM-5-bromo-UTP and PsV-S (0.1 mg/ml). Incubations were at 30°C. Incorporation of ^{3}H-ATP into acid insoluble RNA was determined as described by Chater and Morgan (4).

Analytical ultracentrifugation. Equilibrium density gradient centrifugation of dsRNA in caesium sulphate was carried out in a Beckman Model E ultracentrifuge equipped with a monochromator and a double-beam ultraviolet absorption optical system with photoelectric scanner and multiplexer accessory. Samples, adjusted to a density of about 1.6 g/ml, were placed in cells with double-sector charcoal filled Epon centrepieces and -1° wedge top windows in the AN-F rotor and centrifuged at 34,000 rev/min for 70 h at 25°C. Densities of caesium sulphate solutions were calculated from refractive indices (5) and buoyant densities were calculated as described by Szybalski (6).

RESULTS

Utilisation of 5-bromo-UTP as a substrate for the PsV-S virion RNA polymerase reaction in vitro

Density labelling of DNA has been used widely in studies of the mechanism of DNA replication (7,8). In the replication *in vivo* of *Escherichia coli* DNA (50% A + T) 5-bromouracil incorporates efficiently in place of thymine to produce, after one round of semi-conservative replication, a hybrid (T : BrU) DNA, which banded after centrifugation in equilibrium caesium chloride gradients at a density of 1.760 g/ml, and was readily separated from the original (T : T) DNA, which banded at 1.714 g/ml. It has been

reported that 5-bromo-UTP can replace UTP as a substrate for the conservative transcription of dsRNA *in vitro* by reovirus cores (9).

In order to test whether 5-bromo-UTP can act as a substrate for the PsV-S dsRNA replication reaction *in vitro*, RNA polymerase reaction mixtures containing PsV-S (0.01 mg), ^{3}H-ATP, GTP, CTP and either UTP or 5-bromo-UTP were incubated in parallel until the reactions were complete (18 h). ^{3}H-ATP incorporation into acid insoluble RNA in reactions with UTP was 54,960 cts/min and in reactions with 5-bromo-UTP was 54,490 cts/min. Since PsV-S ds RNA has an A + U content of 47% (2) and its virion RNA polymerase activity requires all four nucleoside triphosphates (4) it is concluded that replication of PsV-S dsRNA *in vitro* occurs as well with 5-bromo-UTP as a substrate as it does with UTP. In confirmation of this conclusion examination of the virus particles, before and after reactions in the presence of 5-bromo-UTP, by equilibrium caesium chloride density gradient centrifugation in the analytical ultracentrifuge showed that RNA synthesis occurred in H particles, which were converted to the more dense P particles, essentially as found before in reactions with UTP (3).

Formation of hybrid (U : BrU) dsRNA

Density labelling of PsV-S dsRNA with 5-BrU was used to investigate the mechanism of the *in vitro* replication reaction. If dsRNA replication took place by a conservative mechanism the product dsRNA in P particles would consist of one molecule of original template (U : U) dsRNA and one molecule of dsRNA labelled in both strands (BrU : BrU) dsRNA; on the other hand with a semi-conservative mechanism the product would consist of two molecules of hybrid (U : BrU) dsRNA. In order to distinguish between these two mechanisms it was necessary to select the low density H particles in which the synthesis of essentially a complete molecule of dsRNA is achieved (Ref. 3 and Fig. 2). Accordingly a preparation of PsV-S was fractionated by sucrose density gradient centrifugation and a fraction consisting of low density H particles plus inactive L particles was incubated with an

RNA polymerase reaction mixture containing 5-bromo-UTP in place of UTP for 18 h. After dialysis to remove excess nucleoside triphosphates, P particles were separated from unreacted L particles by isopycnic caesium chloride density gradient centrifugation. P1 particles had a buoyant density of 1.390 g/ml, compared to the value of 1.387 g/ml found for P1 particles isolated from reactions containing UTP (3). DsRNA, isolated from these more dense P particles, was mixed with caesium sulphate and centrifuged to equilibrium in the analytical ultracentrifuge. The dsRNA formed a single band with a buoyant density of 1.647 g/ml (Fig. 1a); no band of RNA corresponding to unreacted PsV-S dsRNA, buoyant density 1.606 g/ml, could be detected.

DISCUSSION

The formation of a single band of dsRNA with an increased density after PsV-S RNA polymerase reactions in the presence of 5-bromo-UTP and the absence of dsRNA with the buoyant density of the original template dsRNA implies a semi-conservative mechanism for the replication of dsRNA. Moreover the 2.5% increase in density of the brominated dsRNA compared with the unbrominated dsRNA is consistent with that of a hybrid dsRNA containing one brominated and one unbrominated strand, based either on the increase in molecular weight (2.9%), or in comparison with the 2.7% increase in buoyant density on formation of hybrid (T : BrU) dsDNA in *E. coli* after one round of semi-conservative replication in the presence of 5-bromouracil (8).

A diagrammatic representation of semi-conservative replication of dsRNA in virions of PsV-S is shown in Fig. 2. The reaction is envisaged as taking place in two stages (a) displacement of one strand from dsRNA by the RNA strand being newly synthesised and (b) formation of dsRNA using the displaced strand as template. Support for this model is derived from the results of electrophoresis of RNA from medium density H particles in polyacrylamide gels containing 8M-urea which reveal the presence of full length single RNA strands as well as dsRNA (unpublished results of the author).

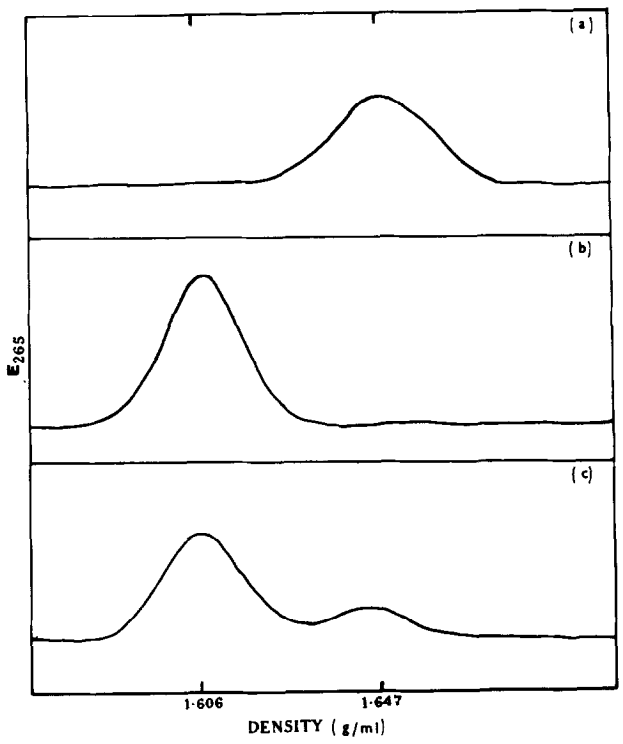

Fig. 1: Equilibrium density gradient centrifugation of dsRNA in caesium sulphate. Scans at 265 nm obtained after centrifuging for 70 h at 34,000 rev/min.; (a) dsRNA obtained from P particles after a PsV-S RNA polymerase reaction in which 5-bromo-UTP was used in place of UTP; (b) unreacted dsRNA from PsV-S; (c) mixture of reacted RNA as in (a) and unreacted RNA as in (b) in the ratio 1:4.

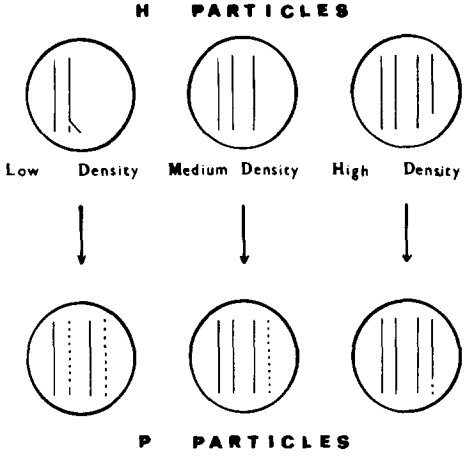

Fig. 2: Diagrammatic representation of semi-conservative replication of PsV-S dsRNA catalysed, within H particles, by the virion associated RNA polymerase. The broken lines represent RNA synthesised during the *in vitro* reaction and do not imply any fragmentation of the product. (For a reaction carried out in the presence of 5-bromo-UTP, the broken line would represent a brominated RNA strand and the unbroken line, an unbrominated RNA strand). The virus capsids are shown as circles.

The dsRNA cannot, in fact, be in a linear conformation within virions as shown in Fig. 2, since the diameter of virus particle is 30 nm (2), whereas the length of a linear PsV-S dsRNA molecule is 415 nm (10); it is probable that the RNA is folded into a highly condensed looped conformation as demonstrated for reovirus dsRNA within virions (11).

PsV-S differs from reovirus and other members of the virus family Reoviridae in several ways; it is smaller, contains fewer dsRNA components and individual dsRNA components are encapsidated separately in different virions. Another difference reported here is that the RNA polymerase of PsV-S virions catalyses the semi-conservative replication of dsRNA within a single virus particle (ds → dsRNA polymerase). In contrast replication of reovirus dsRNA is conservative and takes place in two different kinds of sub-viral particle, (a) transcription of the ten dsRNA segments in virus cores (ds → ssRNA polymerases) with release of the transcripts from the cores and (b) formation of separate nascent sub-virions, containing the ten ssRNA transcripts, which act as templates for the formation of dsRNA by the associated ss → dsRNA polymerase (12,13).

It has been reported that replication of bacteriophage Ø6 dsRNA takes place *in vivo* by a semi-conservative mechanism (14). However there is no evidence that this reaction takes place within virions. Indeed it has been reported that the virion RNA polymerase of phage Ø6 catalyses the "filling in" of short ssRNA tails on predominantly dsRNA molecules (15) and the enzyme is probably similar to the ss → dsRNA polymerase of reovirus nascent sub-virions, the activity observed being the completion of the reaction. The PsV-S dsRNA replication reaction described here is the first report of semi-conservative replication of dsRNA by a virion associated RNA polymerase.

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